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Lipooligosaccharides (LOS) of Some *Haemophilus* Species Mimic Human Glycosphingolipids, and Some LOS Are Sialylated†

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The lipooligosaccharides (LOS) of strains of *Haemophilus ducreyi*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Neisseria lactamica* contain epitopes that are antigenically and structurally similar to carbohydrates present in human glycosphingolipids. LOS from strains of *Haemophilus influenzae* and *H. influenzae* biogroup aegyptius were tested for the binding of monoclonal antibodies (MAbs) that bind to human glycosphingolipids possessing Galβ1-4GlcNAc (MAb 3F11) and Galα1-4Galβ1-4Glc (MAb anti-P^k). In solid-phase radioimmunoassays, the LOS of 18 of 19 *H. influenzae* type b (Hib), 8 of 19 nontypeable *H. influenzae*, and 10 of 20 *H. influenzae* biogroup aegyptius strains bound MAb anti-P^k. The LOS of 13 of 19 Hib, 10 of 16 nontypeable *H. influenzae*, and 2 of 18 *H. influenzae* biogroup aegyptius strains bound MAb 3F11. Neuraminidase treatment of the strains increased the binding of MAb 3F11 by more than twofold in 47% of the *H. influenzae* strains, suggesting that sialic acid occluded the LOS structure recognized by MAb 3F11. The material released from neuraminidase-treated Hib LOS was confirmed to be sialic acid by high-performance anion-exchange chromatography. A recombinant plasmid containing genes involved in Hib LOS biosynthesis directed the expression (assembly) of the 3F11 epitope in *Escherichia coli*. These studies demonstrate that *H. influenzae* and *H. influenzae* biogroup aegyptius express at least two LOS epitopes that are similar to those present in human glycosphingolipids. Sialic acid was present on the LOS of some *H. influenzae* strains and prevented the binding of MAb 3F11 to its epitope. The oligosaccharide portion of sialylated LOS may also resemble sialylated oligosaccharides present in human glycosphingolipids (gangliosides).

The survival of human bacterial pathogens in the host environment requires the complex interaction of multiple factors. The lipooligosaccharides (LOS) of the mucosal pathogens belonging to the genera *Haemophilus* and *Neisseria* mimic host glycolipids, and this mimicry may be an adaptive mechanism that promotes bacterial survival (3, 5, 12, 13, 19, 20). The LOS of pathogenic *Neisseria* species contain epitopes antigenically similar to Galβ1-4GlcNAcβ1-3Gal-R residues present in human paragloboside and other glycosphingolipids (12). The LOS components that contain these epitopes can be sialylated both in vitro and in vivo (3, 13, 14). Sialylation of the Galβ1-4GlcNAcβ1-3Gal-R oligosaccharide in *Neisseria gonorrhoeae* LOS results in an increase in the resistance of *N. gonorrhoeae* strains to the bactericidal activity of normal human serum (17).

We examined LOS purified from *Haemophilus influenzae* type b (Hib), nontypeable (NT) *H. influenzae*, and *H. influenzae* biogroup aegyptius for reactivity with monoclonal antibodies (MAbs) that bind to LOS and glycosphingolipids. The MAbs included 3F11 and 06B4, which bind to Galβ1-4GlcNAc structures, and anti-P^k, which binds to the P^k (Galα1-4Galβ1-4Glc-ceramide) blood group antigen. We also tested whether some *Haemophilus* strains possessed biosynthetic mechanisms for the sialylation of the Galβ1-4GlcNAc epitope. We will present chemical, immunologic, and genetic evidence that strains of Hib, NT *H. influenzae*, and *H. influenzae* biogroup aegyptius contain LOS components that mimic paragloboside and other glycosphingolipids present in human tissues and that some strains of Hib and NT *H. influenzae* are sialylated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Hib A2, *Neisseria meningitidis* serogroup B strain 6275, and *Neisseria gonorrhoeae* F62 have been described previously (13, 14, 18). The other 19 Hib strains were cerebrospinal fluid or nasopharyngeal isolates obtained from our culture collection. Seventeen NT *H. influenzae* strains were obtained from respiratory secretions of adults with chronic lung disease and from middle ear fluids of children with otitis media and hospitalized at Erie County Medical Center or the Childrens Hospital of Buffalo. The 18 *H. influenzae* biogroup aegyptius strains included 10 Brazilian purpuric fever case isolates and were a gift from George Carlone of the Centers for Disease Control. Strains were grown on chocolate agar plates containing 1% IsoVitaleX at 35°C in a 5% CO_2 atmosphere.

Recombinant phage and plasmids. Hib A2 was the source of chromosomal DNA used to construct the recombinant phage EMBLOS-1 (18). When transfected in *Escherichia coli* LE392, EMBLOS-1 encodes the synthesis of an oligosaccharide with an apparent molecular mass of 1,400 (1.4 kDa) on a 4.1-kDa lipopolysaccharide (LPS) structure, producing a chimeric 5.5-kDa LPS band. The 5.5-kDa LPS band contains a 2-keto-3-deoxyoctulosonic acid (KDO) epitope. The added oligosaccharide may render a residue in the *E. coli* deep core accessible to binding by a KDO-specific MAb or may contain a KDO residue (18). The recombinant plasmids pGEMLOS-7, pGEMLOS-5, and pGEMLOS-4

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were constructed by ligating overlapping 2.8-kb *Pst*I-*Sph*I, 5.5-kb *Pst*I-*Hin*dIII, and 7.2-kb *Pst*I-*Bam*HI restriction fragments of the EMBLOS-1 insert into the polylinker of plasmid pGEM3Zf+ (2). In *E. coli* JM109, these plasmids sequentially modify an *E. coli* 4.1-kDa LPS structure, generating chimeric species of 4.5 kDa (pGEMLOS-7), 5.1 kDa (pGEMLOS-5), and 5.5 kDa (pGEMLOS-4). The 5.5-kDa chimeric LPS produced by pGEMLOS-4 also contains a KDO epitope.

MAbs. The specificities and characteristics of anti-LOS MAbs 3F11 and 06B4 have been described previously (15). In brief, both MAbs recognize different configurations of an LOS epitope immunochemically similar to lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-4Galβ1-4Glc) (12). MAb anti-P^k was obtained from Accurate Chemical Co., Westbury N.Y., and recognizes the human P^k glycosphingolipid (Galα1-4Galβ1-4Glc-ceramide).

Preparation of LOS. LOS used for solid-phase radioimmunoassays (SPRIA) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared as described previously (15). LOS from Hib A2 was purified for chemical analysis by the method of Darveau and Hancock (6).

SPRIA. The binding of MAbs to LOS was determined by SPRIA with unheated whole bacteria (1) or purified LOS (15, 16, 23) as antigens. In brief, microtiter plates were coated with LOS at a concentration of 25 μg/ml, incubated with MAbs overnight, and washed. The binding of MAbs to LOS was detected with ^{125}I-goat anti-mouse immunoglobulin M (Kirkegaard and Perry, Gaithersburg, Md.) as described previously (13). The amount of antigen applied to a well may result in day-to-day variations in values obtained by SPRIA. However, replicate samples analyzed by SPRIA have been reported to have standard errors of approximately 7% (23). In this study, the LOS of NT *H. influenzae* 2019 was analyzed four times in two separate experiments, and mean binding values of 2,399 cpm with a standard error of ±120.9 and 2,845 cpm with a standard error of ±92.8 were obtained. All strains were tested for MAb binding in SPRIA in two different experiments, and the correlation coefficient for the values obtained in each experiment was 0.85. For simplicity, the results of one experiment are reported.

N. gonorrhoeae F62 was used as a positive control for MAb 3F11 binding before neuraminidase treatment. *N. meningitidis* 126E, which lacks the LOS component that binds MAb 3F11, was used as a negative control in each experiment. The SPRIA value for strain 126E with MAb 3F11 was 200 cpm; this value was similar to those obtained for wells without antigen and treated with phosphate-buffered saline or neuraminidase (197 to 217 cpm). *N. meningitidis* 6275 was used as a positive control to confirm neuraminidase removal of sialic acid from the Galβ1-4GlcNAcβ1-4Galβ1-4Glc moiety. This structure is constitutively sialylated on this strain when grown on chocolate agar plates. Hence, 3F11 binding increases significantly (at least twofold) to the LOS treated with neuraminidase. A SPRIA value three times higher than the negative control value (600 cpm) was considered positive evidence for antibody binding.

Treatment of LOS with neuraminidase. Whole bacteria (unheated) or purified LOS bound to microtiter wells were treated with various concentrations of *Clostridium perfringens* neuraminidase (Sigma Chemical Co., St. Louis, Mo.) as described previously (13). Treatment of LOS with neuraminidase at 25 mU/ml resulted in optimal binding of MAb 3F11. LOS immobilized on nitrocellulose were also treated with neuraminidase at 25 mU/ml.

SDS-PAGE, Western blotting (immunoblotting), and colony blot analysis. LOS were solubilized and electrophoresed on gels containing 14% acrylamide and 2.5 M urea by a modification of the method of Laemmli (11). Immunoblotting and silver staining were done as described previously (13). The relative molecular masses of the LOS components were estimated by comparison with LPSs isolated from *Salmonella minnesota* R mutants and gonococcal strain PID-2 (15).

To examine the 3F11 epitope for phase variation, we suspended a single 3F11-positive Hib A2 colony in brain heart infusion broth and spread dilutions on chocolate agar plates at 200 to 300 CFU per plate. The colonies were blotted onto nitrocellulose discs and probed with MAb 3F11 as described previously (4, 18). Colonies that did not bind MAb 3F11 were examined subsequently for the ability to express the 3F11 epitope.

Analysis by high-performance anion-exchange chromatography of the products released from neuraminidase-treated Hib LOS. The presence of sialic acid in an *H. influenzae* LOS was detected by a method described previously (13). LOS from Hib A2 (5.9 mg) was treated with 50 mU of neuraminidase (Genzyme, Boston, Mass.) per ml in 25 mM sodium acetate buffer (pH 6.4) for 6 h. As a control, A2 LOS was treated with enzyme that had been heat inactivated. The LOS mixture was passed through a C18 SepPak cartridge (Millipore, Milford, Mass.), and the cartridge was washed with water. The unbound material and the washes were pooled and analyzed for the presence of sialic acid by high-performance anion-exchange chromatography. The chromatography system (Dionex, Sunnyvale, Calif.) consisted of a quaternary gradient pump, a pulsed amperometric detector with a gold electrode, a liquid chromatographic module (LCM-2), a basic postcolumn delivery system, and a Hitachi-Shimadzu C-R3A integrator recorder (13). A CarboPac PA 1 column (4.6 by 250 mm) and a CarboPac PA guard column (3 by 25 mm) (Dionex) were used for the carbohydrate analysis. The enzyme digest and a control sample were analyzed for sialic acid by pulsed amperometric detection at 100 nA with three different conditions (250, 300, and 375 mM NaOH) for the elution of sialic acid. The typical retention time of sialic acid with 375 mM NaOH was 6.9 min. Pentoses, hexoses, and hexosamines were analyzed with 10 mM NaOH as the eluent.

RESULTS

Prevalence and phase variation of the Galβ1-4GlcNAc (3F11) epitope in *Haemophilus* LOS. Strains of Hib, NT *H. influenzae*, and *H. influenzae* biogroup aegyptius were probed with MAbs that recognize carbohydrate antigens both on human glycosphingolipids and on LOS on *N. gonorrhoeae* and *N. meningitidis*. In SPRIA, LOS isolated from 13 of 19 Hib strains bound MAb 3F11 (Fig. 1A). Similarly, 10 of 16 NT *H. influenzae* (Fig. 1B) and 2 of 18 *H. influenzae* biogroup aegyptius strains (Fig. 1C) also bound MAb 3F11. These data suggested that *Haemophilus* LOS are similar to *Neisseria* LOS in that they both contain the 3F11 epitope.

Since *H. influenzae* LOS epitopes are subject to phase variation (20), we tested Hib A2 for phase variation of the 3F11 epitope. A single 3F11-positive colony was subcultured on agar plates; of 1,130 colonies screened, 22 did not bind MAb 3F11 (2.0%). The progeny of a 3F11-negative colony reverted to being 3F11 positive at a rate of 64 of 1,250 colonies tested (5.1%).

Effect of neuraminidase treatment on the binding of MAb

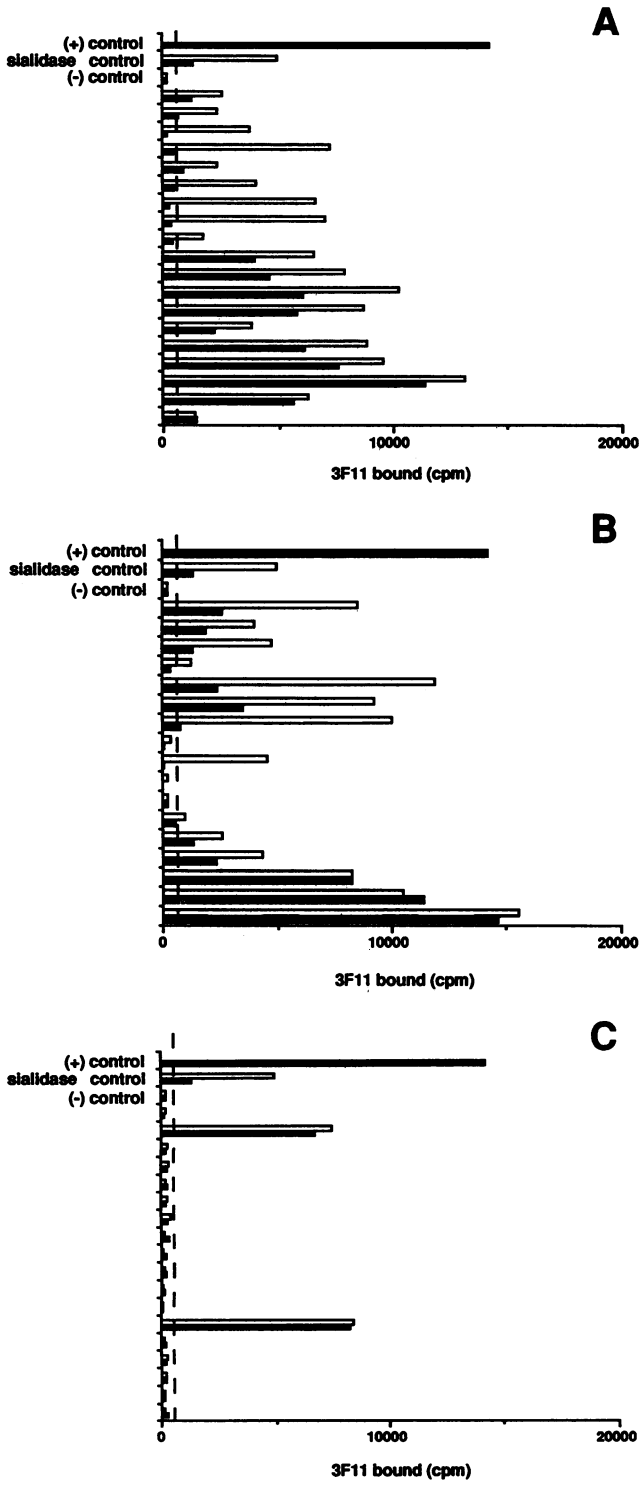


FIG. 1. Binding of MAb 3F11 to LOS on 19 Hib (A), 17 NT *H. influenzae* (B), and 18 *H. influenzae* biogroup aegyptius (C) strains before (■) and after (□) neuraminidase treatment. *N. gonorrhoeae* F62 LOS was the positive control. *N. meningitidis* 126E LOS does not contain the 3F11 epitope and was included as the negative control. *N. meningitidis* 6275 was the sialidase control. The majority of the 3F11 epitopes on 6275 LOS are blocked by sialic acid but can be exposed after treatment with neuraminidase. The broken line indicates 600 cpm, a value which is threefold higher than that obtained with the negative control. Values higher than 600 cpm are considered indicative of MAb 3F11 binding.

TABLE 1. Increase in the binding of MAb 3F11 to LOS on neuraminidase-treated strains of Hib, NT *H. influenzae*, and *H. influenzae* biogroup aegyptius[a]

Strains (no. tested)[b]	No. of strains with the following increase in binding resulting from neuraminidase treatment:			
	None	<20%	21 to 100%	>100%
Hib (19)	1	2	7	9
NT *H. influenzae* (17)	5	0	3	9
H. influenzae biogroup aegyptius (18)	17	1	0	0

[a] Bacteria bound on microtiter wells were treated with neuraminidase and incubated with MAb 3F11 and then with ^{125}I-goat anti-mouse immunoglobulin M as described in the text.

[b] Including strains that bound MAb 3F11 either before or after neuraminidase treatment.

3F11 to LOS. Since sialic acid is sometimes linked to the 3F11 epitope in pathogenic *Neisseria* species, we compared the binding of MAb 3F11 to neuraminidase-treated and untreated *Haemophilus* LOS (Fig. 1A to C). The results shown in Table 1 are representative of the differences in the binding of MAb 3F11 to bacteria before and after treatment with neuraminidase. Treatment with neuraminidase resulted in variable increases (10 to 1,400%) in the binding of MAb 3F11 to Hib. There was a greater than 20% increase in the binding of MAb 3F11 to LOS for 7 of 19 strains of Hib and 3 of 17 strains of NT *H. influenzae*, and there was a greater than 100% increase in the binding of MAb 3F11 to LOS for 18 of 36 strains of Hib or NT *H. influenzae* (Table 1). Only 2 of the 18 *H. influenzae* biogroup aegyptius strains bound MAb 3F11 before treatment with neuraminidase (Fig. 1C). Little change in MAb 3F11 binding was seen after treatment of these strains with neuraminidase (Table 1). These results suggested that some of the Hib and NT *H. influenzae* strains express the 3F11 epitope, that the epitope is expressed in various amounts, and that the difference in the expression of the epitope can be due in part to modification of the epitope by sialic acid.

Binding of MAb 06B4 to *Haemophilus* LOS. The binding of MAb 06B4 to neuraminidase-treated and untreated bacteria was also tested. None of the strains bound MAb 06B4 before they were treated with the enzyme (data not shown). However, 3 of 19 Hib strains bound MAb 06B4 after treatment with the enzyme; none of the NT *H. influenzae* or *H. influenzae* biogroup aegyptius strains bound MAb 06B4 after the enzyme treatment (data not shown). These data suggested that *Haemophilus* LOS differ from *Neisseria* LOS in that the former rarely contain the 06B4 epitope.

Detection of sialic acid in Hib LOS. To confirm that the increased binding of MAb 3F11 to neuraminidase-treated LOS was due to the release of sialic acid, we analyzed neuraminidase-treated LOS from Hib A2 by high-performance anion-exchange chromatography (Fig. 2). Sialic acid was the major sugar detected in the hydrolysate obtained from neuraminidase-treated A2 LOS (Fig. 2). The amount of sialic acid in A2 LOS was estimated to be approximately 0.4% by weight. No material was released from LOS that had been incubated with heat-inactivated neuraminidase.

Detection of the components in Hib A2 LOS that bind MAb 3F11. To determine which components of A2 LOS bound MAb 3F11, we separated Hib A2 LOS by SDS-PAGE, transferred the LOS to nitrocellulose, and probed it with MAb 3F11. In a similar analysis in a previous study, we had

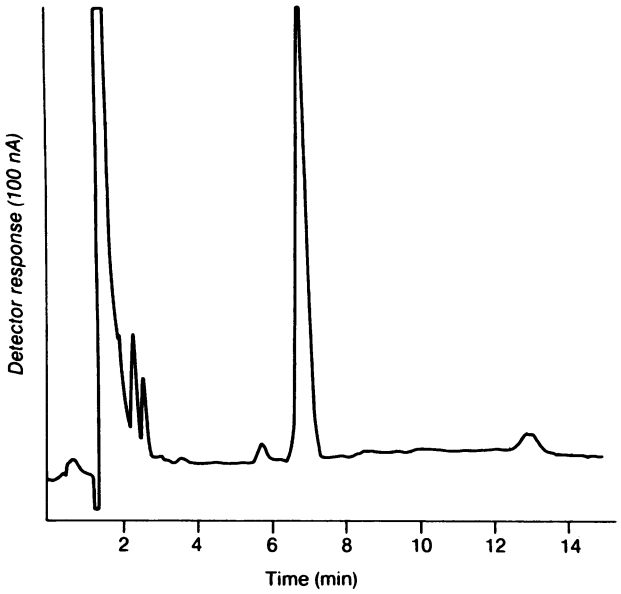


FIG. 2. Sialic acid release from A2 LOS. The products released by neuraminidase digestion of A2 LOS were analyzed by high-performance anion-exchange chromatography (eluate, 375 mM NaOH; flow rate, 1 ml/min; amperometric detection, 100 nA). The hydrolysate was also analyzed with other elution conditions (250 and 300 mM NaOH), and the carbohydrate released from the LOS had the same retention time as authentic sialic acid. No pentoses, hexoses, or *N*-acetylhexosamines were detected in the hydrolysate (eluate, 10 mM NaOH; flow rate, 1 ml/min; amperometric detection, 30 nA).

noted that 0.5 to 1 μg of LOS from pathogenic *Neisseria* species was sufficient to detect the binding of MAb 3F11 (15). However, for detection of the LOS components that bind MAb 3F11 in Hib A2 LOS, electrophoresis of 5 to 10 μg of LOS was required. One A2 LOS band immobilized on nitrocellulose and treated with heat-inactivated neuraminidase bound MAb 3F11; the band had an apparent molecular mass of approximately 3.4 kDa (Fig. 3). After treatment of the immobilized LOS with neuraminidase, MAb 3F11 bound to two bands. One band migrated with an apparent molecular mass of 3.4 kDa, and the other band migrated at 3.7 kDa. These data confirmed that the treatment of A2 LOS with neuraminidase exposed more of the 3F11 epitope and suggested that the 3.4-kDa LOS component (3F11 positive) was the precursor for the sialylated 3.7-kDa LOS component (3F11 negative).

Genetic evidence that Hib assembles a Galβ1-4GlcNAc

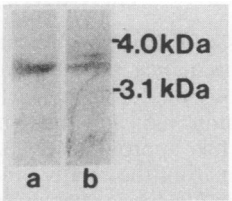


FIG. 3. Neuraminidase treatment of Hib A2 LOS immobilized on nitrocellulose. Hib A2 LOS components were separated by SDS-PAGE and transferred to nitrocellulose, and the nitrocellulose was incubated with intact or heat-inactivated neuraminidase, with MAb 3F11, and with anti-mouse immunoglobulin M. Lanes: a, A2 LOS treated with heat-inactivated neuraminidase; b, A2 LOS treated with intact neuraminidase.

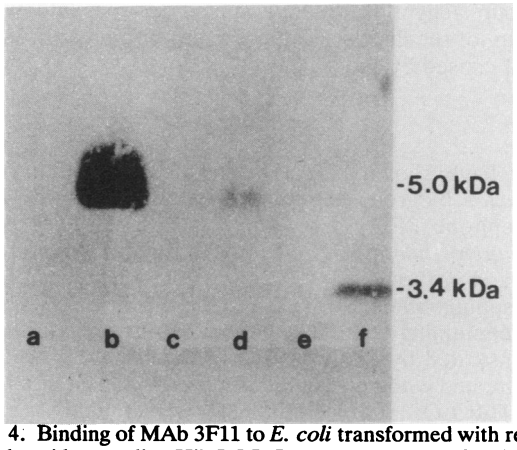


FIG. 4. Binding of MAb 3F11 to *E. coli* transformed with recombinant plasmids encoding Hib LOS. Lanes: a, LOS purified from *E. coli* JM109 transformed with pGEM3Zf+; b, LOS isolated from *E. coli* LE392 transfected with a clone that contained an A2 chromosomal restriction fragment similar to pGEMLOS-5; c, LOS isolated from *E. coli* JM109 transformed with pGEMLOS-7; d, LOS isolated from *E. coli* JM109 transformed with pGEMLOS-5; e, LOS isolated from *E. coli* JM109 transformed with pGEMLOS-4; f, LOS isolated from *H. influenzae* A2.

epitope. We previously reported the isolation of a recombinant phage, EMBLOS-1, that contains a 14-kb Hib A2 insert and assembles an oligosaccharide with an apparent molecular mass of 1.4 kDa on a 4.1-kDa core LPS structure of *E. coli* LE392, revealing a KDO epitope (18). Recombinant plasmids containing overlapping restriction fragments derived from EMBLOS-1 also direct the sequential assembly of oligosaccharides of 0.4, 1.0, and 1.4 kDa on an *E. coli* JM109 4.1-kDa LPS species (2). In Western blots, JM109 transformed with plasmid pGEMLOS-5 contained a chimeric 5.1-kDa LPS band that bound MAb 3F11 (Fig. 4). Recombinant phage derived from an A2 EMBL3 library (2) also bound MAb 3F11 and contained inserts that had similar restriction maps and hybridized to pGEMLOS-5. LPS isolated from JM109 transformed with vector pGEM3Zf+ or recombinant plasmids pGEMLOS-7 (encoding the 0.4-kDa modification) and pGEMLOS-4 (encoding the 1.4-kDa modification) did not bind MAb 3F11. These data suggested that pGEMLOS-5 encoded enzymes that assembled or exposed the 3F11 epitope in *E. coli* by modifying the R_a core LPS and that the additional modification(s) encoded by pGEMLOS-4 occluded the 3F11 epitope.

Prevalence of the P^k epitope (Galα1-4Galβ1-4Glc-ceramide). The results of SPRIA analysis with MAb anti-P^k are shown in Table 2. The P^k epitope was present on 18 of 19 Hib strains, 7 of 17 NT *H. influenzae* strains, and 9 of 18 *H. influenzae* biogroup aegyptius and strains. The presence or absence of the P^k epitope on strains of *H. influenzae*

TABLE 2. Binding of MAb anti-P^k to Hib, NT *H. influenzae*, and *H. influenzae* biogroup aegyptius strains

Strains (no. tested)	No. of strains showing	
	MAb anti-P^k binding	No MAb anti-P^k binding
Hib (19)	18	1
NT *H. influenzae* (17)	7	10
H. influenzae biogroup aegyptius (18)	9	9

biogroup aegyptius did not appear to correlate with the severity of the disease (Brazilian purpuric fever or conjunctivitis) caused by the strains.

DISCUSSION

We have shown that Hib, NT *H. influenzae*, and *H. influenzae* biogroup aegyptius strains express an MAb-defined epitope (3F11) that is also present in the LOS of strains of *N. gonorrhoeae*, *N. meningitidis*, and *Haemophilus ducreyi* and in the Galβ1-4GlcNAcβ1-3Gal residues of human glycosphingolipids (5, 12). The component of *Haemophilus* LOS containing the 3F11 epitope also can be sialylated, as demonstrated by (i) the increased binding of MAb 3F11 to LOS treated with neuraminidase, (ii) the release of sialic acid from Hib LOS after neuraminidase treatment of the LOS, and (iii) the binding of MAb 3F11 to an additional LOS component only after neuraminidase treatment of the LOS. The LOS of the majority of Hib and NT *H. influenzae* strains expressed the 3F11 epitope, but this expression occurred infrequently among the LOS of *H. influenzae* biogroup aegyptius strains. The epitope defined by MAb 3F11 was occluded by sialic acid on Hib and NT *H. influenzae* strains at similar frequencies.

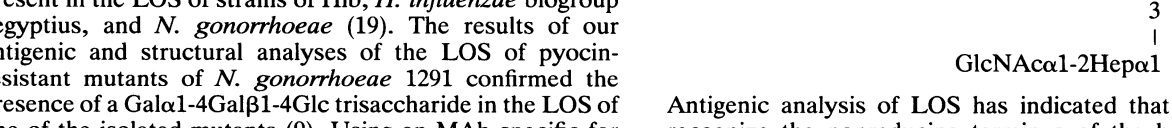
We had previously reported the isolation of a recombinant phage, EMBLOS-1, that assembled or exposed a KDO epitope in *E. coli* (18). Subcloning and deletion analysis of this phage showed that it contains at least three LPS-modifying loci within a 7.2-kb *Pst*I-*Bam*HI restriction fragment (2). A plasmid subclone containing a 5.5-kb *Pst*I-*Hin*dIII fragment and two of the modifying loci assembled (or exposed) the 3F11 epitope when the plasmid was transformed into *E. coli* K-12 strain JM109. *E. coli* transformed with a plasmid containing a 2.8-kb *Pst*I-*Sph*I fragment and responsible for at least one LPS modification did not bind 3F11; however, the presence of the *Pst*I-*Sph*I fragment was necessary for the formation of the 3F11 epitope (data not shown) and might have provided a substrate for subsequent epitope assembly. The 3F11 epitope was occluded and a KDO epitope was exposed in *E. coli* transformed with plasmid pGEMLOS-4, which contains the entire *Pst*I-*Bam*HI fragment. Thus, phage EMBLOS-1 may contain loci responsible for the assembly or exposure of two LOS epitopes present in many Hib strains, and one of these epitopes may resemble a human glycosphingolipid.

The presence of a glycosphingolipidlike epitope in *Haemophilus* strains is consistent with the results reported in a recent study by Virji et al. (19). This group showed that an epitope antigenically similar to a Galα1-4Gal disaccharide is present in the LOS of strains of Hib, *H. influenzae* biogroup aegyptius, and *N. gonorrhoeae* (19). The results of our antigenic and structural analyses of the LOS of pyocin-resistant mutants of *N. gonorrhoeae* 1291 confirmed the presence of a Galα1-4Galβ1-4Glc trisaccharide in the LOS of one of the isolated mutants (9). Using an MAb specific for the P^k blood group antigen (Galα1-4Galβ1-4Glc-ceramide), we confirmed that the P^k-like residue is present in other strains of *H. influenzae* and *H. influenzae* biogroup aegyptius.

In a previous study with a few strains of Hib and NT *H. influenzae*, we had been unable to detect any binding of MAb 3F11 to LOS (5). This lack of detection might have been due to the relatively larger amount of the 3F11 epitope present on *N. meningitidis* and *N. gonorrhoeae* LOS than on *Haemophilus* LOS. For *N. meningitidis* and *N. gonorrhoeae* LOS, gel samples containing 0.5 to 1.0 μg resulted in the detection of the 3F11 epitope (5, 14). In contrast, for *Haemophilus* species, it was necessary to use between 5 and 10 μg of sample. The lower concentration of the epitope in untreated Hib LOS is reflected by a single band binding MAb 3F11, compared with the large number of bands (up to eight in tricine SDS-PAGE systems) present in untreated *H. influenzae* LOS (2, 18). Treatment of the SDS-PAGE-separated, nitrocellulose-immobilized A2 LOS with neuraminidase resulted in a second 3F11-positive band, suggesting that only one band in A2 LOS had been sialylated (Fig. 3). The difference in the sensitivity of detection of the 3F11 epitope on the *Neisseria* and *Haemophilus* LOS could also have been due to (i) the smaller amount of the 3F11 epitope expressed in the untreated LOS used for electrophoresis, (ii) the loss of some of the 3F11-positive band during preparation of the sample for electrophoresis or during phenol-water purification of the LOS (data not shown), or (iii) a difference in the conformation of the epitope. The 0.4% sialic acid detected in purified A2 LOS by anion-exchange chromatography indicates that the concentration of the sialylated 3F11 epitope is approximately 10-fold lower than that described for a comparable sample of meningococcal strain 6275 LOS (13). Further chemical analysis will be required to determine the sialylation characteristics of *Haemophilus* LOS.

Previous studies with strains of *H. ducreyi*, *N. gonorrhoeae*, and *N. meningitidis* indicated that MAb 06B4 has a specificity similar to that of MAb 3F11 (5, 12, 13). However, the absence of binding of MAb 06B4 to most of the 3F11-positive *Haemophilus* LOS in this study suggested that the basic structure of the 3F11/06B4 epitope on *Haemophilus* LOS may be different from that which occurs on other mucosal pathogen LOS. This difference may be related to the difference in the binding of MAb 3F11 and MAb 06B4 to epitopes of branching or linear carbohydrates on glycosphingolipids (12) and to epitopes of sialylated meningococcal LOS (13). Four strains of Hib bound MAb 06B4 after the bacteria were treated with neuraminidase (data not shown), suggesting that the 06B4 epitope exists in Hib strains but that it can be completely occluded by sialylation. Further antigenic and chemical analyses will be required to determine the difference in the 3F11 and 06B4 epitopes (component size, sialylation differences, structure, etc.) in Hib, NT *H. influenzae*, and *Neisseria* strains.

Structural analysis of the MAb 3F11- and 06B4-defined LOS component(s) of *N. gonorrhoeae* F62 (21, 22) has shown that lacto-*N*-neotetraose is present at the nonreducing end of the longer oligosaccharide, as shown below:



Antigenic analysis of LOS has indicated that both MAbs recognize the nonreducing terminus of the branch which contains the lacto-*N*-neotetraose (12, 22). The data presented in this study indicate that the LOS component binding MAb 3F11 is approximately 3.4 kDa. This size is somewhat different from the relative molecular mass of the LOS components in *Neisseria* species and in *H. ducreyi* that bind MAb 3F11 (4.5 kDa) (5, 13, 14). This discrepancy could be due to a number of factors, including (i) a difference in the carbohydrate structures of the epitopes; (ii) a difference in the amount or location of phosphorylation, which could result in differences in the migration of LOS in SDS-PAGE; and (iii) a difference in the structures of the lipid A moieties

of *Neisseria* species and Hib. The reason for the difference in the apparent sizes of the 3F11-related sialylated and nonsialylated LOS on *Neisseria* species and Hib can only be resolved after complete chemical and structural definition of the 3F11 epitope of *Haemophilus* species.

The function of the sialylated LOS structure in *Haemophilus* species is not known. It has been demonstrated that gonococci with sialylated LOS are more resistant to the bactericidal effect of normal human serum (17). However, Kurtana and coworkers (10) showed that the growth of Hib Eagan in the presence of CMP-*N*-acetylneuraminic acid (NANA) had no effect on the serum sensitivity of the strain. Our studies suggest that, unlike gonococci (8, 14) but like serogroup B and C meningococci (8, 13), some *Haemophilus* species can synthesize the necessary sialic acid precursor in culture medium without any supplementation. It is possible that the *Haemophilus* strain tested in the earlier study (10) did not have the LOS component required for exogenous sialylation during growth in the presence of CMP-NANA or that the strain was already sialylated (endogenously) and therefore was not affected by growth in the presence of CMP-NANA. It will be of interest to determine whether the mechanisms of sialylation of LOS in *Haemophilus* species are similar to those in *Neisseria* species.

Haemophilus and *Neisseria* species include pathogens that cause disease in human mucosal tissues. First, the bacteria colonize nasopharyngeal or genital surfaces, and then they disseminate either to other mucosal surfaces or into the circulation. Although there is little interspecies conservation among the protein and capsular antigens, recent studies have revealed that there are similar and, in some cases, identical LOS epitopes present among the *Haemophilus* and *Neisseria* pathogens (5, 12, 19). These LOS epitopes are also present in the carbohydrate moieties of human paragloboside and of other human glycosphingolipids (3, 12, 19), suggesting that the LOS in these bacteria might be part of a common mechanism of survival for bacteria located in mucosal environments. For example, the carbohydrate residues of human glycoproteins that bind to receptors involved in recycling desialylated glycoproteins often contain Galβ1-4GlcNAc (7). The sialylated and desialylated LOS components on bacteria may bind to eukaryotic receptors, enhancing the invasive potential of these microbes. Stable isogenic LOS mutants of *Haemophilus* and *Neisseria* species would be helpful in resolving the role of these structures in pathogenesis.

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